

RELATED APPLICATIONS

1. INTRODUCTION FIELD OF THE INVENTION

2. BACKGROUND OF THE INVENTION

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cultured with 10 $\mu\text{g/ml}$ each of anti- α/β , anti-CD3, CD40-Ig or MR1. RNA synthesis was determined as described in Figure 1. Results presented are the arithmetic means of triplicate cultures \pm s.d., and are representative of 3 such experiments.

Figure 6. MR1 and CD40-Ig recognized the same molecule expressed on activated T_h .

Panel A: Activated T_h were fluorescently stained with MR1 or control Ig. To evaluate if CD40-Ig and MR1 competed for binding to activated T_h , graded concentrations of MR1 or control hamster Ig (anti- α/β TCR) were added together with anti-CD40 (20 $\mu\text{g/ml}$). After incubation for 20 minutes at 4°C, the samples were washed and incubated with FITC-conjugated, mab anti-human IgG₁. Results are representative of 3 such experiments.

Panel B: Proteins from [³⁵S]-methionine-labelled, activated T_h were immunoprecipitated with MR1 (10 $\mu\text{g/sample}$) or CD40-Ig (10 $\mu\text{g/sample}$) and resolved by PAGE and fluorography. Results presented are representative of 2 such experiments.

Figure 7. Binding of CD40-Ig to human cell lines. A variety of human T-cell lines were exposed to biotin-labelled CD40-Ig, and binding was evaluated by flow cytometry.

Figure 8.

Panel A: Nucleotide sequence of CD40 cDNA from *Stamenkovic et al.*, (1989) *EMBO J.* 8:1403-1410. The transmembrane region is underscored.

Panel B: Schematic diagram of a plasmid that may be used to express CD40-Ig. The amino acid sequences at the site of fusion of Δ CD40 is shown below the diagrammed portion of CD40 (SEQ ID NO:3).

5. — DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for ~~A~~substantially purified CD40CR receptor; for soluble ligands of CD40CR, including antibodies as well as fusion molecules comprising CD40; and for methods of controlling B-cell activation.

For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

- (i) ligands that bind to CD40CR;
 - (ii) methods used to characterize CD40CR;
 - (iii) preparation of purified CD40CR;
 - (iv) uses of ligands that bind to CD40CR;
- and
- (v) uses of CD40CR.

5.1—LIGANDS THAT BIND TO CD40CR

Ligands that Bind to CD40CR

The present invention provides for soluble ligands of CD40CR, including (i) fusion molecules comprising at least a portion of CD40 protein and (ii) antibodies or antibody fragments.

The term “soluble”, as used herein, indicates that the ligands of the invention are not permanently associated with a cell plasma membrane. Soluble ligands of the invention may, however, be affixed to a non-cellular solid support, including a lipid, protein, or carbohydrate molecule, a bead, a vesicle, a magnetic particle, a fiber, etc. or may be enclosed within an implant or vesicle.

The ability of such a ligand to bind to CD40CR may be confirmed by demonstrating that the ligand binds to the same protein as CD40-Ig (infra) or MRI (infra).

The ligands of the invention may be comprised in pharmaceutical compositions together with a suitable carrier.

5.1.1—FUSION MOLECULES

Fusion Molecules

The present invention provides for soluble fusion molecules that are ligands of CD40CR. Such fusion molecules comprise at least a portion of CD40 protein attached to a second molecule. The portion of CD40 preferably lacks the CD40 transmembrane domain. A portion of CD40 protein which may be used according to the invention is defined as any portion which is able to bind to CD40CR, for example, such a portion may be shown to bind to the same protein as MR1 or CD40-Ig.

Second molecules which may be used include peptides and proteins, lipids, and carbohydrates, and, in preferred embodiments of the invention, may be an immunoglobulin molecule, or portion thereof (such as an Fv, Fab, F(ab')₂, for Fab' fragment) or CD8, or another adhesion molecule, such as B7. The second molecule may be derived from either a non-human or a human source, or may be chimeric. The second molecule may also be an enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound.

The fusion molecules of the invention may be produced by chemical synthesis or, preferably, by recombinant DNA techniques.

For example, a nucleic acid sequence encoding at least a portion of CD40 protein may be combined with a nucleic acid sequence encoding a second molecule in a suitable expression vector, and then expressed in a prokaryotic or, preferably, eukaryotic expression system, such as a yeast, baculovirus, or mammalian expression system, including transgenic animals.

Alternatively, at least a portion of CD40 protein may be expressed using recombinant DNA techniques and then may be chemically conjugated to a second molecule.

Fusion molecules comprising CD40 may be purified from preparative mixtures using electrophoretic techniques or affinity chromatography using ligand that binds to either CD40 or to the second molecule. Ligands that bind to CD40 include, but are not limited to, anti-CD40

antibodies such as G28-5, as produced by the hybridoma having accession number HB9110 and deposited with the American Type Culture Collection, and CD40CR, described more fully in sections 5.2 and 5.3, infra. If the second molecule is an immunoglobulin or immunoglobulin fragment, an affinity column comprising anti-immunoglobulin antibody may be used; if the second molecule comprises an F_c fragment, a protein A column may be used.

According to a preferred embodiment of the invention, a portion of CD40 may be produced using a nucleic acid sequence that encodes a CD40 protein that is truncated upstream from the transmembrane domain. Such a nucleic acid sequence may be prepared by digesting a plasmid containing cDNA encoding CD40 antigen, such as that described in *Stamenkovic et al.*, (1989), *EMBO J.* 8:1403-1410, with PstI (P) and Sau 3A (S3) restriction enzymes. The resulting P/S3 fragment may be subcloned into the same plasmid digested with P and Bam HI (B), to produce a truncated CD40 gene (see Figure 8).

In particular, nonlimiting, embodiments of the invention, an expression vector used to produce ligands containing at least a portion of CD40 as well as immunoglobulin sequence may preferably comprise a virally-derived origin of replication, a bacterial origin of replication, a bacterial selectable marker, and eukaryotic promoter and enhancer sequences separated from DNA sequences encoding an immunoglobulin constant region by restriction endonuclease sites which allow subcloning of DNA sequences encoding at least a portion of CD40, followed by a polyadenylation signal sequence (see Figure 8.b.).

In a specific embodiment of the invention, the truncated CD40 gene may be subcloned into an immunoglobulin fusion plasmid, such as that described in *Aruffo et al.*, 1990, *Cell* 61:1303-1313, using an Mlu I and B digest, to form plasmid pCD40-Ig, which encodes the fusion molecule CD40-Ig (see Figure 8). CD40-Ig fusion protein may then be produced by transfecting the pCD40-Ig plasmid into COS cells to form a transient expression system. CD40-Ig produced may be collected from the COS cell supernatant and purified by protein A column chromatography as described in *Aruffo et al.*, 1990, *Cell* 161:1303-1313.

5.1.2. ANTIBODIES

Antibodies

The soluble ligands of the invention may comprise antibody molecules, monoclonal antibody molecules, or fragments of these antibody molecules which contain an antigen combining site that binds to CD40CR. Such ligands may further comprise a second molecule which may be protein, lipid, carbohydrate, enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound and may be linked to the antibody molecule or fragment.

Where the ligand is a monoclonal antibody, or a fragment thereof, the monoclonal antibody can be prepared against CD40CR using any technique which provides for the production of antibody molecules by continuous cell lines in culture. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497) as well as other techniques which have more recently become available, such as the human B-cell hybridoma technique (Kozbar et al., 1983, *Immunology Today* 4:72) and EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) and the like are within the scope of the present invention.

Antibody fragments which contain the idiotype of the molecule could be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be generated by treating the antibody molecule with pepsin; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment; the $F(ab')_2$ fragment which can be generated by treating the antibody molecule with papain; and the 2Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent to reduce the disulfide bridges.

The present invention also provides for chimeric antibodies produced by techniques known in the art, such as those set forth in *Morrison et al.*, (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81:6851-6855 or *European Patent Application No. 85305604.2*, publication No. 0173494 by *Morrison et al.*, published March 5, 1986.

CD40CR may be characterized by its ability to bind to ligands such as CD40, fusion molecules comprising CD40, and antibodies directed toward CD40CR.

As discussed in greater detail infra, several techniques were used to characterize CD40CR. For example, CD40-Ig and MR1 were shown to recognize the same 39 kD molecule. Both CD40-Ig and MR1 were found to immunoprecipitate a 39 kD protein from radiolabelled T_h lysates (Figure 5b). Further, immunoprecipitation of the 39 kD protein with CD40-Ig removed the antigen recognized by MR1 from T_h lysates.

5.2.2. ABILITY TO STIMULATE B-CELLS

CD40CR may also be characterized by its ability to stimulate B-cell cycle entry, proliferation, and differentiation.

For example, plasma membrane (PM) from activated (PM^{Act}) but not resting (PM^{rest}) T_h cells was found to induce B-cell RNA synthesis (Figure 1a); this induction, indicative of B-cell activation, was not; affected by antibodies such as anti-LFA-1, anti-CD4, anti-ICAM-1. CD40-Ig or MR1, however, were found to be able to inhibit PM^{Act} -induced B-cell activation, as shown in Figure 1b and Figure 6.

The induction of B-cell activation may be measured by techniques such as [³H]-uridine incorporation into RNA (as B-cells differentiate, RNA synthesis increases), or by [³H]-thymidine incorporation, which measures DNA synthesis associated with cell proliferation. For optimal measurement of the effect of CD40CR on B-cell proliferation, interleukin-4 (IL-4) may be added to the culture medium at a concentration of about 10 ng/ml.

Alternatively, B-cell activation may be measured as a function of immunoglobulin secretion. For example, CD40Cr, in substantially purified form, or as present in PM, or otherwise, may be added to resting B-cells together with IL-4 (10 ng/ml) and IL-5 (5 ng/ml). After three days of culture, an additional volume of culture medium may be added. On a day 6 of culture, supernatant (SN) from individual cultures may be harvested and quantitated for IgM and IG₁ as described in *Noelle et al., (1991) J. Immunol. 146:1118-1124.*

5.2.3 CELLULAR DISTRIBUTION

CD40CR may also be characterized by its cellular distribution. For example, CD40-Ig was observed to bind to activated, but not resting T_h1, as assessed by flow cytometry (Figure 3). Furthermore, CD40-Ig was observed to bind to Jurkat cells, HSB2 cells, and activated T-cells from human peripheral blood, but did not appear to bind significantly to CEM cells, HPBALL cells, or murine thymoma cells.

For example, and not by way of limitation, the presence of CD40CR on a particular cell type ("test cells") may be evaluated by flow cytometry as follows. Test cells may be tested in parallel with resting (negative control) and activated (positive control) T_h cells. All cells may be incubated at a concentration of about 1×10^5 cells/50 μ l with ligand (e.g. CD40-Ig or MR1) for 20 minutes at 4°C, followed by FITC-conjugated anti-ligand antibody. Propidium iodide may be added to all samples to a final concentration of 2 μ g/ml. Flow cytometric analysis may then be performed, for example on a BD FACSCAN. After positive gating of cells by forward versus side scatter, and by red negativity (for propidium iodide exclusion), and the log green fluorescence of viable cells may be ascertained.

5.3 PREPARATION OF PURIFIED CD40CR

Preparation of Purified CD40CR

The present invention provides for substantially purified CD40CR. Such CD40CR may be prepared from cells bearing CD40CR, such as activated helper T-cells, Jurkat, and HSB2 cells, by the following method.

Plasma membranes may be prepared from appropriate cells, such as activated T_h1 cells, by discontinuous sucrose gradient sedimentation, as described in *Noelle et al., 1991, J. Immunol. 146:1118-1124*. CD40CR may then be isolated by dissociating the crude membrane extract with mild detergent, and then performing size exclusion chromatography followed by either affinity chromatography using appropriate ligands (e.g., MR1 or CD40-IG) bound to a solid support, immunoprecipitation (e.g. by CD40-IG or MR1), and/or gel electrophoresis.

The resulting protein may be expected to have a molecular weight of about 39 kD.

The present invention provides for a soluble CD40CR (i.e., cell-free) which may be comprised in pharmaceutical compositions together with a suitable carrier. It further provides for CD40CR which is linked to a second molecule which may be a peptide, protein, lipid, carbohydrate, enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound.

The present invention further provides for substantially purified CD40CR which has been prepared by chemical synthesis or recombinant DNA techniques. For example, the gene for CD40CR may be isolated by inserting cDNA prepared from activated helper T-cells into the λ gt10 expression system, and then screening with MR1 or CD40-Ig binding to identify CD40CR-expressing clones. Alternatively, cDNA prepared from activated helper T-cells may be transfected into COS cells, the supernatants of which may be screened with MR1 or CD40-Ig to identify CD40CR producers. The gene for CD40CR may be then used to express CD40CR using expression systems in the art.

5.4 — USES OF LIGANDS THAT BIND TO CD40CR

Uses of Ligands that Bind to CD40CR

The present invention provides for methods of controlling B-cell activation that utilize ligands that bind to CD40CR. In particular, it provides for a method of inhibiting B-cell activation comprising exposing a mixture of B-cells and T_h cells to an effective concentration of ligand that binds to CD40CR. Ligands that may be used are described supra ~~in section 5.1.~~ The method of the invention may be practiced in vitro or in vivo. An effective concentration refers to a concentration of a ligand that inhibits B-cell activation, measured by any technique known in the art (including those set forth ~~in section 5.2,~~ supra) by at least about 30 percent, and preferably by about 75 percent. According to a preferred, specific, non-limiting embodiment of the invention, CD40-Ig may be used as ligand, in which invention, CD40-Ig may be used as ligand, in which case an effective concentration may be at least about 10

μg/ml. In another specific, nonlimiting embodiment of the invention, the monoclonal antibody MR1 may be used, in which case an effective concentration may be at least about 10 μg/ml. If the method is practiced in vivo, an effective concentration of ligand may refer to plasma concentration of ligand or to a local concentration. For example, it may be desirable to inhibit B-cell activation in a localized area in order to limit the effects on the immune system as a whole.

In particular embodiments, the invention provides for a method of treating a subject suffering from a disorder associated with B-cell activation, comprising administering to the subject a therapeutic amount of ligand that binds to CD40CR. A subject may be a non-human or, preferably, a human animal.

Disorders associated with B-cell activation include, but are not limited to, allergy (including anaphylaxis); autoimmune conditions including drug induced lupus, systemic lupus erythematosus, adult rheumatoid arthritis, juvenile rheumatoid arthritis, scleroderma, Sjogren's Syndrome, et c.; and viral diseases that involve B-cells, including Epstein-Barr infection, and retroviral infection including infection with a human immunodeficiency virus.

Because it has been suggested that B-cell activation is associated with the induction of human immunodeficiency virus replication from latency, it may be desirable to administer the ligands of the invention to HIV positive individuals who have not yet developed AIDS or ARC.

Ligands may be administered, in a suitable pharmaceutical carrier, by any method known in the art, including intravenous, intraperitoneal, subcutaneous, intrathecal, intraarticular or intramuscular injection, and oral, intranasal, intraocular and rectal administration, and may be comprised in microspheres, liposomes, and/or sustained released implants.

A therapeutic amount of ligand is defined as an amount which significantly diminishes the deleterious clinical effects of B-cell activation, and may vary among ligands used and conditions treated. If CD40-Ig is used, therapeutic concentration may be about 10 μg/ml either

systemically (plasma concentration) or locally. If MR1 is used, a therapeutic concentration may be about 10 µg/ml either systemically (plasma concentration) or locally.

In a further embodiment of the invention, the above methods may utilize a ligand comprising a toxin or antimetabolite such that T_h cells are killed or damaged and B-cell activation is decreased as a result of T_h cell destruction.

The ligands of the invention may also be used to label activated T cells, a technique which may be useful in the diagnosis of T cell disorders. To this end, ligand comprising an enzyme, radioisotope, fluorescent compound or other detectable label may be exposed to T cells in vitro or in vivo and the amount of binding may be quantitated.

The ligands of the invention may also be used to deliver substances, e.g. growth factors, to be activated T-cells.

5.5 — USES OF CD40CR

Uses of CD40CR

The present invention provides for methods of controlling B-cell activation that utilize CD40CR or a molecule comprising CD40CR, prepared as described ~~in section 5.3, supra~~. In particular, it provides for a method of promoting B-cell activation comprising exposing B-cells to an effective concentration of CD40CR. The method may be practiced in vivo or in vitro. An effective concentration refers to a concentration of receptor that induces B-cell activation, measured by any technique known in the art (including those set forth ~~in section 5.3, supra~~) by at least about 30 percent. In specific, nonlimiting embodiments of the invention, the concentration of CD40CR may be about 10 µg/ml locally or systemically.

In particular embodiments, the invention provides for a method of treating a subject suffering from an immunodeficiency disorder associated with diminished humoral immunity, comprising administering to the subject a therapeutic amount of CD40CR. A subject may be a non-human or, preferably, a human animal.

Immunodeficiency disorders associated with diminished humoral immunity include acquired immunodeficiency syndrome, immunodeficiency associated with malignancy or cachexia, iatrogenic immunodeficiency caused, for example, by chemotherapy or radiation therapy, as well as genetic disorders involving humoral immunity.

CD40CR may be administered, in a suitable pharmaceutical carrier, by any method known in the art, including intravenous, intraperitoneal, subcutaneous, intrathecal, intraarticular, or intramuscular injection, and oral, intranasal, intraocular, and rectal administration and may be comprised in micropsheres, liposomes, and/or sustained release implants.

A therapeutic amount of CD40CR for CD040 is defined as that amount which increases immunoglobulin production by at least about 30 percent.

In a further embodiment, CD40CR may be conjugated to a toxin, and then administered to a subject under circumstances in which it would be preferable to destroy B-cells that express CD40. Examples of such circumstances include patients receiving organ transplants or suffering from multiple myeloma or another B-cell malignancy, or from autoimmune disease.

CD40CR may also be used to label B-cells expressing CD40, a technique which may be useful in the diagnosis of B-cell disorders. To this end, receptor linked to an enzyme, radioisotope, fluorescent compound or other detectable label may be exposed to B-cells in vivo or in vitro and the amount of binding may be quantitated.

CD40CR may also be used to deliver molecules that are linked to it to B-cells.

~~6. EXAMPLE A NOVEL RECEPTOR, CD40CR, ON ACTIVATED HELPER T-CELLS BINDS CD40 AND TRANSDUCES THE SIGNAL FOR COGNATE ACTIVATION OF B-CELLS~~

~~6.1. MATERIALS AND METHODS~~

~~6.1.1. ANIMALS~~

EXAMPLES

Example 1: A Novel Receptor, CD40CR, On Activated Helper T-Cells Binds CD40
And Transduces the Signal for Cognate Activation of B-Cells

Materials And Methods

Animals

Female DBA/2J mice (Jackson Laboratories, Bar Harbor, MA) were used for the preparation of filler cells to support the growth of T_h clones and in the preparation of resting B-cells.

~~6.1.2. HELPER T-CELL CLONES (T_H)~~

Helper T-Cell Clones (Th)

D1.6, a I-A^d-restricted, rabbit Ig-specific Th1 clone (*Kurt-Jones et al., (1987), J Exp Med* 166:1774-1787) was obtained from Dr. David Parker, University of Mass. at Worcester. D1.6 will be referred to herein as Th1.

6.1.3. ACTIVATION OF T_H BY ANTI-CD3

Activation of Th by Anti-CD3

Th1 were cultured (8×10^6 /well) in cluster wells (6 well, Corning, NY) coated with 40 μ g/4 ml of PBS/well with anti-CD3 for 16 hours, as described in (Noelle et al., (1991) *J. Immunol.* 146:1118-1224).

6.1.4. PREPARATION OF T_h PLASMA MEMBRANES

Preparation of Th Plasma Membranes

Plasma membranes were prepared by discontinuous sucrose gradient sedimentation, as described in (Noelle *et al.*, (1991) *J. Immunol.* 146:1118-1124).

6.1.5 PREPARATION OF RESTING B-CELLS

Preparation of Resting B Cells

Resulting splenic B-cells were prepared by sedimentation on discontinuous ~~Percoll~~ PERCOLL[®] gradients, as described in (Defrano et al., (1982) *J. Exp. Med.* 155:1523). Cells isolated from the 70-75% (density of 1.087-1.097) ~~Percoll~~ PERCOLL[®] interface were typically >95% mIg⁺, had a uniform, low degree of near forward light scatter and were unresponsive to Con A.

6.1.6 ANTIBODIES

Antibodies

The following mabs were purified by ion exchange HPLC from ascites fluid of mice which had been irradiated and bone marrow reconstituted: anti-CD3:145-2C11 (Leo et al., (1987) *Proc. Natl. Acad. Sci. USA* 84: 1374-1378); anti- α,β :H57-597; anti-CD4: GK1.5 (Wilde et al., (1983) *J. Immunol.* 131:2178-2183); anti-ICAM: YN1/1.7.4 (Prieto et al., (1989) *Eur. J. Immunol.* 19:1551-1557); anti-LFA-1: FD441.8 (Sarmiento et al, (1982) *Immunol. Rev.* 68:135); and anti-rat/hamster κ chain:RG-7 (Spring, (1982) *Hybrid.* 1:257-273).

6.1.7 PREPARATION OF THE CD40 RECOMBINANT

GLOBULIN (CD40-Ig)

Preparation of the CD40 Recombinant Globulin (CD40-Ig)

The CD40 fusion protein was prepared by digesting a plasmid containing a cDNA encoding the CD40 antigen (Stamenkovic and Seed, (1989) *EMBO J.* 8:1403-1410) with the restriction enzyme Pst I (P) and Sau 3A (S3). This P/S3 fragment was subcloned into the same plasmid digested with P and Bam H1 (B). This allowed the preparation of the CD40 Δ which encoded a CD40 protein truncated upstream from the transmembrane domain. The DNA fragment encoding a CD40 Δ was then subcloned into the immunoglobulin fusion plasmid (Aruffo et al. (1990), *Cell.* 61:1301-1313) using a MluI and B digest. The CD40-Ig fusion

protein was produced by transient transfection in COS cells and purified on a protein A column as described in (Aruffo et al., (1990) Cell. 61:1303-1313).

6.1.8. LYMPHOKINES

Lymphokines

Interleukin 4 (IL4): Recombinant mouse IL4 was generously provided by Drs. C. Maliszewski and K. Grabstein, Immunex Corporation, Seattle, WA.

Interleukin 5 (IL5): Recombinant mouse IL5 was purchased from R&D Research, Sarrento, CA.

6.1.9. INDUCTION OF B-CELL RNA SYNTHESIS BY ACTIVATED T_h PLASMA MEMBRANES

Induction of B-Cell RNA Synthesis by Activated Th Plasma Membranes

3 X 10⁴ resting B-cells were cultured in 50 µl of cRPMI in A/2 microtiter wells (Costar, Cambridge, MA). To these wells, 0.5 µg of T_h1 or T_h2 membrane protein was added. From 42-48 hrs, wells, were pulsed with 2.5 µCi of ³H-uridine (New England Nuclear, Boston MA), harvested, and the radioactivity determined by liquid scintillation spectroscopy. The results were expressed as cpm/culture +/-s.d.

6.1.10 INDUCTION OF B-CELL IMMUNOGLOBULIN SECRETION BY ACTIVATED T_h PLASMA MEMBRANES AND LYMPHOKINES

Induction of B-Cell Immunoglobulin Secretion by Activated Th Plasma Membranes and Lymphokines

Resting B-cells were cultured as described above. To culture wells, 0.5 µg of T_h1 membrane protein, IL4 (10 ng/ml) and IL5 (5 ng/ml) were added. On day three of culture, an additional 50 µl of cRPMI was added. On day three of culture, an additional 50 µl of cRPMI

Results

Effect of Monoclonal Antibodies on the Induction of B Cell RNA Synthesis by PMAct

In order to define the cell surface molecules that mediated the induction of B-cell cycle entry by PM^{Act}, mabs to T_h membrane proteins were added to cultures of PM^{Act} and B-cells. PM^{Act} induced B-cell RNA synthesis eight-fold over that observed with PM^{rest}) (Figure 1a). The addition of anti-LFA-1, anti-CD4, anti-ICAM-1, alone, or in combination, did not inhibit the induction of B-cell RNA synthesis by PM^{Act}.

~~6.2.2. CD40 Ig INHIBITED T-INDUCED B-CELL CYCLE ENTRY, DIFFERENTIATION AND PROLIFERATION~~

CD40-Ig Inhibited T-Induced B-Cell Cycle Entry, Differentiation and Proliferation

In the human system, it had been shown that anti-CD40 mab induced B-cell proliferation (*Clark and Lane, (1991) Ann. Rev. Immunol. 2:97-127*) thereby implicating CD40 as an important triggering molecule for B-cells. To determine if CD40 was involved in the induction of B-cell RNA synthesis by PM^{Act}, a soluble fusion protein of the extracellular domains of human CD40 and the Fc domain of human IgG₁ (CD40-Ig) was added to cultures of PM^{Act} derived from Th1 and Th2 were prepared and used to stimulate B-cell RNA synthesis. The addition of CD40-Ig to culture caused a dose-dependent inhibition of B-cell RNA synthesis that was induced by PM^{Act} from Th1 and Th2 (Fig. 1b). Half-maximal inhibition of B-cell RNA synthesis induced by PM^{Act} from Th1 and Th2 was about 5 µg/ml CD40-Ig. A CD7E-Ig fusion protein (*Damle and Aruffo, (1991) Proc. Natl. Acad. Sci. USA 88:6403-6407*) was without effect even when used at 25 µg/ml.

To investigate whether CD40-Ig inhibited the activation of B-cells by T-independent activators, B-cells were cultured in the presence of LPS and CD40-Ig. On day 2, RNA synthesis was assessed (Fig. 1c). CD40-Ig was ineffective at inhibiting B-cell activation by LPS, yet inhibited the response of B-cells to PM^{Act}.

In the presence of PM^{Act}, IL4 and IL5, B-cells polyclonally differentiated to produce Ig (Hodgkin *et al.*, (1990) *J. Immunol.* 145:2025-2034; (Noelle *et al.*, (1991) *J. Immunol.* 146:1118-1124). To evaluate the requirements for CD40 signalling in this process, CD40-Ig was added at the initiation of culture, or on subsequent days of culture. The addition of CD40-Ig (Fig. 2a) at the initiation of culture inhibited greater than 95% of polyclonal IgM and IgG₁ production compared to control levels in its absence. In contrast, the addition of CD40-Ig on day 1 and 2 of culture showed little, if any, inhibitory effect on IgM and IgG₁ production. These data indicated that after 24 hours, signalling via CD40 is no longer essential for the differentiation of B-cells to Ig secretion.

Data thus far indicated that CD40 was implicated in the activation of B-cells by PM^{Act}. Studies were performed in order to ensure that CD40 was also involved in the activation of B-cells by intact, viable, activated T_h. T_h1 were activated for 16 hours with insolubilized anti-CD3, harvested and irradiated. The irradiated T_h1 were cultured with B-cells in the presence of IL4 and B-cell proliferation was determined on day 3 of culture. An exogenous source of IL4 was required to achieve B-cell proliferation with T_h1, because T_h1 do not produce IL4 (Noelle *et al.*, (1989) *J. Immunol.* 143:1807-1814). CD40-Ig inhibited the induction of B-cell proliferation by irradiated T_h in a dose-dependent manner, similar to that observed with PM^{Act} (Fig. 2b). The negative control, CD7E-Ig, exerted no appreciable effect.

6.2.3 CD40-Ig DETECTED A MOLECULE EXPRESSED ON ACTIVATED, BUT NOT-RESTING T_h

CD40-Ig Detected a Molecule Expressed on Activated, but not Resting T_h

To investigate whether activated T_h1 express a binding protein for CD40, resting and activated (16 hours) T_h1 were stained with CD40-Ig or CD7E-Ig, followed by FITC-anti-HigG. Binding of CD40-Ig was assessed by flow cytometry (Fig. 3). T_h1 that were activated for 16 hours with anti-CD3, but not resting T_h1, stained 56% positive with CD40-Ig, but not with the control CD7E-Ig. To identify the CD40-Ig binding protein, T_h1 proteins were biosynthetically labelled with [³⁵S]-methionine/cysteine and proteins immunoprecipitated with CD40-Ig or CD7E-Ig. The immunoprecipitated proteins were resolved by SDS-PAGE and fluorography

hamster mabs or CD40-Ig. Two hamster mabs, anti- α/β TCR and α -CD3 did not inhibit the activation of resting B-cells by PM^{Act}. In contrast, MR1 or CD40-Ig inhibited B-cell activation (Fig. 6).

6.3—DISCUSSION

Discussion

The data show that blocking of prominent T_h surface molecules (LFA-1, CD4, ICAM-1, CD3, α/β TCR) with mabs did not impede the capacity of activated T_h to induce B-cell cycle entry. In contrast, CD40-Ig or a maB specific to the CD40 binding protein, blocked T_h-dependent B-cell activation in a dose-dependent manner. Furthermore, the CD40 binding protein was identified as a 39 kD protein that is selectively expressed on the membranes of activated, but not resting T_h. Both CD40-Ig and a mab specific to the 39kD CD40 binding protein blocked B-cell activation by PM^{Act}.

Although a number of membrane proteins have been implicated in T_h-dependent B-cell signalling, evidence presented herein dismisses the contribution of some molecules (LFA-1, CD4, CD3, α/β TCR, ICAM-1) and implicates CD40 as the B-cell receptor for cognate signalling by T_h. Data show that CD40-Ig and a mab specific to the CD40 binding protein inhibits T_h-dependent B-cell activation.

The ligand for CD40 is a 39 ~~Kd~~ kD protein that is expressed on activated, but not resting T_h. Biochemical studies indicate that the 39kD protein is a single chain molecule since electrophoretic migration was not influenced by reducing agents. Based on the functional studies presented in this study, both activated T_h1 and T_h2 express the 39 kD CD40 binding protein. This is consistent with the functional studies that show both T_h1 and T_h2 induce B-cell cycle entry. In an attempt to further characterize the 39 kD protein, cDNA encoding CD proteins in the MW range of 39kD (CD 53, CD27 and CD69) were transiently transfected into COS cells and the cells were tested for CD40-Ig binding. None of the transfected COS cells expressed proteins that bound CD40-Ig. It is therefore suspected that the 39 kD protein is not one of these CD proteins.

The biochemical basis for signal transduction between T_h and B-cells has been elusive. The identification of CD40 as the signal transducing molecule for T cell help focusses focuses attention on specific biochemical pathways known to be coupled to the CD40 molecule. CD40 is a member of the nerve growth factor receptor (NGFR) family by the virtue of the presence of four cysteine-rich motifs in its extracellular region. Signaling through CD40 by mab has been shown (*Uckun et al., (1991) J. Biol. Chem. 266:17478-17485*) to involve the activation of tyrosine kinases resulting in the increased production of inositol trisphosphate and the activation of at least four distinct serine/threonine kinases. Based on information obtained from signaling through other members of the NGF receptor family, it is anticipated that interaction between activated T_h and B will result in many of the same biochemical processes.

7. — EXAMPLE: BINDING OF CD40 Ig TO HUMAN T-CELL LINES

Example 2: Binding of CD40 Ig to Human T-Cell Lines

For immunofluorescence binding studies, CD40 Ig fusion protein was conjugated with biotin using biotin-succinimide (Sigma). Flow cytometry analysis was then performed by tow-step staining using phycoerythrin (PE)-streptavidin (Bectin-Dickinson) with a Coulter Epics C instrument. Representative results of screening multiple T cell lines is presented below. The Jurkat and HSB2 cell lines were found to bind specifically, whereas other T cell lines including CEM, HPBALL, and murine thymoma did not bind the CD40 Ig fusion protein (Fig. 7).

Various publications are cited herein which are hereby incorporated by reference in their entirety.

The hybridoma identified in this application as MR1 was deposited on May 22, 1992 with the American Type Culture Collection, ATCC, International Depository Authority, 12301 Parklawn Drive, Rockville, Maryland 20852 10801 University Boulevard, Manassas, VA 20110-2209, in compliance with the Budapest Treaty, and accorded Accession Number ATCC HB 11048. All restrictions as to the availability to the public of the hybridoma cell line MR1 will be irrevocably withdrawn upon issuance of a United States Patent to this application. Also, access to the MR1 cell line will be available to the Commissioner during the pendency of

this patent application or to one determined by the Commissioner to be entitled to such cell line under 37 C.F.R. §1.14 and 35 U.S.C. §122.